Freedom of movement

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Ulrich Rass

Holliday junction resolvases lock dynamic DNA four-way junctions into specific structural conformations for symmetric DNA cleavage. Single-molecule studies now reveal that resolvases can relax their grip, enabling Holliday junction conformer transitions and branch migration in the enzyme-bound form.

Robin Holliday's DNA four-way junctions[1] are central intermediates of homologous recombination and homology-directed DNA double-strand break repair. They arise by strand-swapping between two homologous DNA helices at DNA breaks. Following DNA ligation, mature Holliday junctions (HJs) covalently link recombining molecules. Their removal is then mediated by a topoisomerase-dependent pathway (HJ dissolution) or by an operationally defined class of structure-specific nucleases known as HJ resolvases[2] (HJ resolution). Resolvases avidly bind HJs and are quite unique in their ability of introducing symmetrically-related incisions across the branch-point, cleanly separating two nicked duplex molecules. Free HJs are highly dynamic, but crystal-structure analyses of resolvase-HJ complexes show extensive protein-DNA interactions that appear to block any movement, unless the complex dissociates[2]. Zhou *et al*.[3] now use single-molecule experiments to reveal nearly unencumbered HJ dynamics in the enzyme-bound form. This unexpected behavior was observed for HJs bound by a range of viral, bacterial, fungal and human enzymes. The authors infer an evolutionary conserved, partially-dissociated (PD) state, in which resolvases hang on to the substrate while allowing DNA movements that may alter recombination outcomes (**Fig. 1a**).

HJs adopt an open planar, cross-shaped form with arms pointing towards the corners of a square. In the presence of metal ions, which quench electrostatic repulsion of DNA backbone phosphates, the arms undergo coaxial stacking, forming two continuous helices with antiparallel orientation in the so-called stacked X-structure[2]. With a choice of two stacking partners, two alternative conformers of the stacked X-structure exist (**Fig. 1b**). Single-molecule fluorescence resonance energy transfer (smFRET) approaches enable analyses of HJ dynamics in real time using synthetic HJs with two of the four arms labeled with fluorophores. Previous studies have detected FRET level transitions consistent with X-structure conformer exchange and branch migration, and deduced that each proceeded through an open planar intermediate[4,5] (**Fig. 1b**).

In their current study[3], Zhou *et al.* asked whether these HJ dynamics cease upon resolvase binding – as might be expected from co-crystal structures – by analyzing individual complexes by smFRET using internal reflection microscopy. Free HJs exhibited fast FRET level changes consistent with conformer exchange and branch migration. Addition of resolvases suppressed these fluctuations and produced distinct FRET readings compatible with enzyme-imposed HJ distortions revealed in co-crystal structures. While these findings suggest that resolvases capture and "freeze" the instantaneous HJ conformer equilibrium, occasional FRET level transitions consistent with conformer exchange and branch migration in the enzyme-bound form were also observed. When these transitions occurred, brief visits and short phases of rapid FRET level oscillations with signal intensities similar to those produced by free HJs were detected. Having employed conditions to carefully minimize the possibility of dissociation and re-binding, the authors postulate a novel PD binding mode, in which unencumbered HJ dynamics are possible without resolvases having to let go of their substrate. This implies multivalent protein-DNA interactions that allow resolvases to toggle between binding modes: in the fully-bound mode HJs are molded into extensive DNA-binding interfaces and immobile; in PD mode, resolvases disengage partially, enabling unrestricted HJ structural transitions. This model is supported by accelerated transitions of enzyme-bound HJs when the DNA-binding capacity of resolvases was compromised by mutation or conditions of high ionic strength.

The PD-mode concept expands the possibilities for HJ processing. On the one hand, crossover/non-crossover decisions, dependent on HJ conformer choice, may be regulated even after resolvase engagement. On the other hand, partial dissociation from HJs represents an effective way for sequence-specific resolvases to stay attached to the target when branch migration is required. This could prevent futile protein-DNA complex formation of sequence-specific resolvases such as RuvC, which recognizes HJs in a purely structure-specific manner. Other resolving enzymes show mild or no sequence preference[2], and it remains to be seen whether the PD mode might be physiologically relevant when instantaneous DNA cleavage is possible. It will be important to substantiate the proposed PD state by defining the DNA-binding interface, which is not addressed in the current study. It would appear that a substantial number of contacts seen in resolvase-HJ co-crystals must be broken in the PD mode. The authors envision that dimeric resolvases likely disengage at least one subunit, and it should be possible to test this hypothesis using smFRET and mutational analysis. These studies could also lead to important insights regarding an unresolved question: How do branch migration motor proteins, exemplified by *E. coli* RuvA and RuvB cooperate with HJ resolvases such as RuvC? While

genetic and biochemical studies show that RuvAB-catalyzed branch migration is directly linked with RuvC-mediated HJ resolution[6], it is unclear how RuvC might be able to continuously sample the branch point as part of a RuvABC resolvasome and mediate resolution once a cognate DNA sequence moves into position. The PD mode offers an elegant solution, allowing the necessary freedom of movement for branch migration and conformer exchange whilst maintaining protein-DNA contacts. Besides recombination, HJ resolvase multivalency may be important in the context of DNA replication stress. Replication fork reversal following replication stalling generates four-way DNA junctions equivalent to HJs[7]. Reversed forks are emerging targets for HJ resolvases[8] and a PD binding-mode could be key for coordinating their actions with the many other proteins involved in fork processing. The elegant single-molecule studies by Zhou *et al*.[3] bring new movement into our view of branched-DNA processing, and investigating more complex branch-migration and resolution reactions in real time is an exciting prospect for the future.

Ulrich Rass is *at the Genome Damage and Stability Centre*
University of Sussex, Falmer, Brighton, UK.
email: u.w.rass@sussex.ac.uk

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Figure 1. A new partially-dissociated (PD) state of resolving enzymes enables unrestricted HJ dynamics. (**a**) HJ dynamics govern genetic exchange during homologous recombination and homology-directed DNA repair. Branch migration determines the length of heteroduplex DNA, which gives rise to gene conversion. The choice of HJ resolution cleavage plane decides

whether recombination is accompanied by crossing-over, the exchange of flanking markers. (**b**) HJs transition between two possible stacked X-structures through an open intermediate (middle). In the stacked X-structure, two exchanging DNA strands pass from one helical axis to the other, while two non-exchanging strands continue along the outer boundary of each axis. Conformer transitions make exchanging stands become non-exchanging strands, and vice versa, such that different pairs of strands present themselves to the resolving enzymes. Therefore, conformer equilibrium determines crossover formation. *Below*, while resolvases lock HJs into specific structural conformations, the PD mode of binding allows unencumbered HJ dynamics.